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Modulation of atherosclerosis in ApoE-deficient mice by gp130 signalling independent of STAT3

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Highlights

- Deregulated gp130 activation in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice suppresses atherosclerosis.
- Modulation of aortic plaque formation by gp130 is independent of STAT3 signalling.
- Gp130/STAT3 signalling promotes plaque macrophage infiltrates and SAA production.
- Gp130 signalling in bone marrow cells does not contribute to atherosclerosis.

Modulation of atherosclerosis in ApoE-deficient mice by gp130 signalling independent of STAT3

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Abstract

Objective: Interleukin (IL)-6 is a key modulator of the acute phase response (APR), and while both are implicated in atherosclerosis, the pathological role of specific IL-6 signalling cascades is ill-defined. Since IL-6 employs the cytokine receptor gp130 to primarily activate the STAT3 pathway, here we evaluate whether gp130-dependent STAT3 activation modulates atherosclerosis.

Methods: High-fat diet-induced atherosclerosis was established in *ApoE*$^{-/-}$ mice crossed with *gp130*$^{F/F}$ knock-in mice displaying elevated gp130-dependent STAT3 activation and production of the APR protein, serum amyloid A (SAA). Also generated were *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice displaying genetically-normalised STAT3 activation and SAA levels, and bone marrow chimeras involving *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice. At 10 weeks post high-fat diet, aortic atherosclerotic lesions, including the presence of CD68^{+} macrophages, and plasma lipid and SAA profiles, were assessed.

Results: Aortic plaque development and plasma triglyceride levels in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice were significantly reduced (3-fold, $P<0.001$) compared to *ApoE*$^{-/-}$ littermates. By contrast, in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice, atherosclerotic plaques contained augmented CD68^{+} macrophage infiltrates, and plasma SAA levels were elevated, compared to *ApoE*$^{-/-}$ mice. Atherosclerotic lesion development and plasma triglyceride levels in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice were comparable, despite a significant ($P<0.05$) reduction in macrophage numbers in lesions, and also plasma SAA levels, in *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice. Aortic plaque development and plasma triglyceride levels were comparable in *ApoE*$^{-/-}$ mice reconstituted with *gp130*$^{F/F}$:*ApoE*$^{-/-}$ (ApoE$^{F/F:ApoE}$) or *ApoE*$^{-/-}$ (ApoE^{ApoE}) bone marrow cells.

Conclusions: Deregulation of gp130/STAT3 signalling augments the APR and macrophage infiltration during atherosclerosis without impacting on the development of aortic plaques.

1. Introduction

Atherosclerosis is the predominant underlying pathology of cardiovascular disease, and its prevalence world-wide has reached epidemic proportions due to the adoption of a Western life-style comprising a diet high in cholesterol and saturated fats.[1] In addition to clinical studies, mouse disease models have been extensively used to gain insights into the cellular and molecular pathogenesis of atherosclerosis, with mice deficient in ApoE (*ApoE*$^{-/-}$), a key apolipoprotein involved in the regulation of lipoprotein metabolism, being the most widely-investigated model due to their hyper-susceptibility to atherosclerosis when fed a high-fat, Western-style diet.[2] Collectively, these studies have revealed that immune deregulation, leading to chronic inflammation in the arterial wall, as well as lipid metabolism affecting circulating cholesterol and triglyceride levels, play a causal role in promoting atherosclerosis.[3-6]

Interleukin-6 (IL-6) is a potent immunomodulatory cytokine with pro- and anti-inflammatory properties involved in the regulation of the acute phase response (APR), as well as modulation of lipid homeostasis and vascular remodelling.[7,8] These complex and pleiotropic actions of IL-6 within the cardiovascular system most likely explain, at least in part, the contradictory roles that have been attributed to IL-6 in the development of atherosclerosis in several mouse models. For instance, exogenous addition of IL-6 to *ApoE*$^{-/-}$ mice potentiates disease severity,[9] and genetically modified *ApoE*$^{-/-}$ mice unable to elicit the hepatic-driven APR via IL-6 are protected against atherosclerosis,[10] indicating a pro-atherogenic role for IL-6 and the APR. Conversely, IL-6 deficiency in the susceptible LDL receptor knock-out (*Ldlr*$^{-/-}$) mice impaired the production of the key APR protein serum amyloid A (SAA) but had no effect on the development of atherosclerosis,[11] and others have suggested an anti-atherogenic role for IL-6 based on the augmented development of atherosclerotic lesions in either *ApoE*$^{-/-}$ or *ApoE*$^{-/+}$ mice deficient in IL-6.[12,13] While these observations imply that IL-6 and its downstream signalling pathways are crucial determinants in disease pathogenesis, the identity of gp130 signalling pathways which promote these diverse and contrasting atherogenic actions for IL-6 remains ill-defined.

IL-6 signals via the common signal-transducing receptor subunit gp130, which it shares with several related cytokines including IL-11, IL-27, oncostatin M, ciliary neurotrophic factor, and leukemia inhibitory factor among others.[14] Ligand-induced dimerization of gp130 leads to activation of gp130-associated Janus kinases (JAKs), which tyrosine phosphorylate (pY) gp130 to primarily facilitate the recruitment of signal transducer and activator of transcription (STAT) 3, and to a lesser extent STAT1 and the SH2-containing tyrosine phosphatase 2 (SHP2), the latter of which mediates activation of extracellular-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K)/Akt signalling pathways.[14] In addition, the pY_{757} amino acid of murine gp130 protein (pY_{759} of human gp130) plays a crucial role in attenuation of gp130/STAT3 signalling by recruiting suppressor of cytokine signalling (SOCS)3 to gp130, which leads to proteosomal degradation of the receptor signalling complex.[15]

Several *in vivo* approaches designed to artificially modulate STAT3 activity, either indirectly by targeting SOCS3 or more directly by modulating STAT3 expression levels, have not only led to conflicting observations regarding the role of STAT3 during atherogenesis, but also have not addressed which upstream cytokine receptor system is responsible for activation of STAT3.[16-19] In this current study, we have used *gp130*$^{F/F}$ knock-in mice displaying endogenous gp130-dependent STAT3 hyper-activation[20,21] on the *ApoE*$^{-/-}$ background to elucidate the role of this signalling axis in atherosclerosis. Here, we report that *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice are largely protected against atherosclerosis when fed a high-fat diet, which is concomitant with specific alterations to their lipid profile. The development of atherosclerotic lesions was comparable in both *gp130*$^{F/F}$ and compound mutant *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice[21] on the *ApoE*$^{-/-}$ background, despite the latter displaying genetically-normalised endogenous STAT3 signalling, decreased plasma SAA levels, and reduced macrophage infiltrates in plaques. Furthermore, bone marrow chimeras revealed that the suppressed atherosclerosis was independent of altered gp130 signalling in haemopoietic-derived (i.e. immune) cells. Collectively, these data suggest that the suppression of atherosclerosis in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice is independent of the endogenous gp130/STAT3/SAA axis.

2. Materials and methods

2.1 Mice and experimental induction of atherosclerosis

The *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Stat3*$^{-/+}$ mice on a mixed 129Sv×C57BL/6 background[20,21] were back-crossed 10 generations onto a C57BL/6 background prior to mating with C57BL/6 *ApoE*$^{-/-}$ mice (provided by P. Tipping, Monash University, Australia). Male and female mice aged 8 weeks were fed an atherogenic high-fat, Western-style diet comprising 0.15% cholesterol and 21% milk fat (Specialty Feeds, Glen Forrest, Australia) ad libitum for 10 weeks. All mice were housed under specific pathogen-free conditions, and experiments were approved by the Monash Medical Centre 'A' Monash University Animal Ethics Committee.

2.2 Bone marrow reconstitution

Bone marrow reconstitution experiments were performed as described previously.[22] Briefly, unfractionated bone marrow cells (5×10^6) from *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice were injected intravenously into lethally γ-irradiated (2×5.5Gy) 8 week old *ApoE*$^{-/-}$ mice. At 8 weeks post-transfer, mice were fed a high-fat diet for 10 weeks. The successful reconstitution of mice was determined by measuring spleen weights and circulating blood platelet counts, as described previously.[23]

2.3 Assessment of atherosclerosis

Aortas were dissected and perfused to allow for quantification of atherosclerotic lesions in Oil Red O-stained aortic root sections as described previously.[24] Immunohistochemistry was performed with an anti-CD68 antibody (clone FA-11, kindly provided by P. Tipping, Monash University, Australia) as described previously.[25]

2.4 Plasma profiling

Blood was collected after overnight fasting, and total plasma cholesterol and triglyceride levels were determined.[24] SAA protein levels were determined by ELISA (Biosource, Camarillo, California, USA) as per the manufacturer's instructions.

2.5 Statistical analyses

Statistical analyses were performed using GraphPad Prism for Windows version 5.0. The normality of data was assessed using the D'Agostino and Pearson omnibus K2 normality test. Where appropriate, parametric (one-way ANOVA, student t-test) or nonparametric tests (Kruskal Wallis, Mann-Whitney U test) were used to determine differences between genotypes. A $P<0.05$ was considered statistically significant. Data are expressed as the mean±standard error of the mean (SEM).

3. Results

3.1 Amelioration of atherosclerosis in ApoE-deficient mice with altered gp130 signalling

The *gp130*$^{F/F}$:*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ littermate control mice (8 weeks old) were fed a high-fat, Western-style diet for 10 weeks. Although the *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice weighed slightly less than their *ApoE*$^{-/-}$ counterparts prior to being fed a high-fat diet, the mean body weight increase of both *gp130*$^{F/F}$:*ApoE*$^{-/-}$ or *ApoE*$^{-/-}$ mice was comparable over the 10 week course of high-fat diet (Fig. 1A). Notably, the percentage of the aortic root occupied by plaques (red-stained areas) was significantly reduced (~3-fold) in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$ mice (*gp130*$^{F/F}$:*ApoE*$^{-/-}$, 9.925±1.357% versus *ApoE*$^{-/-}$, 23.960±1.744 %, $P<0.001$) (Fig. 1B,C). Similarly, further quantification indicated that the total plaque area was significantly smaller in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ versus *ApoE*$^{-/-}$ mice (*gp130*$^{F/F}$:*ApoE*$^{-/-}$, 0.05906±0.008962mm^2 versus *ApoE*$^{-/-}$, 0.1733±0.01772 mm^2, $P<0.001$) (Fig. 1D). The amelioration of atherosclerotic plaque formation in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice was specifically in response to high-fat diet, since there was no significant plaque formation in either *gp130*$^{F/F}$:*ApoE*$^{-/-}$ or *ApoE*$^{-/-}$ mice fed a normal chow diet for up to 6 months (data not shown).

While alterations to lipid metabolism can influence the pathogenesis of atherosclerosis,[5,26] the role of gp130 signalling in modulating systemic lipid levels during atherosclerosis remains controversial.[12,13,27] Serum lipid profiling of high-fat diet-fed *gp130*$^{F/F}$:*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice revealed a significant reduction in the level of serum triglycerides in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice (Fig. 2A), whereas total, HDL and LDL cholesterol levels were comparable between the two genotypes (Fig. 2B-D). To control for any effects of the high-fat diet, similar serum levels of total, HDL and LDL cholesterol, and triglycerides, were observed in 9 month old *gp130*$^{F/F}$:*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice fed a normal chow diet (Supplemental Fig. 1). These data therefore indicate that alterations to endogenous gp130 signalling specifically influences the systemic production of triglycerides in response to high-fat diet, the suppression of which correlated with reduced atherosclerosis in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice.

3.2 Suppressed atherosclerosis in gp130$^{F/F}$:ApoE$^{-/-}$ mice does not correlate with reduced aortic plaque macrophage infiltration or plasma SAA levels

To examine whether the reduction in aortic plaque development in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice correlated with suppressed inflammation, we next performed immunohistochemistry with the CD68 antibody to detect infiltrating macrophages, which represent the majority of inflammatory cell infiltrates in atherosclerotic plaques. However, the extent of macrophages in atherosclerotic lesions in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice appeared higher than that in lesions from *ApoE*$^{-/-}$ mice fed a high-fat diet (Fig. 3A). Further quantification also confirmed that the percentage of CD68^{+} macrophages in atherosclerotic plaques was significantly higher in high-fat diet-fed *gp130*$^{F/F}$:*ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$ mice (Fig. 3B). Since the gp130-mediated APR, in particular SAA production, has been linked to the recruitment of macrophages into aortic plaques,[10] we also examined whether the high-fat diet affected SAA levels in the plasma of *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice. Indeed, SAA plasma levels were significantly increased by ~20-fold in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice compared to *ApoE*$^{-/-}$ mice (Fig. 3C). Collectively, these observations suggest that the suppressed atherosclerosis in high-fat diet-fed *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice occurs independently of the gp130-mediated APR and macrophage infiltration in atherosclerotic plaques.

3.3 Activation of gp130/STAT3 signalling in gp130$^{F/F}$*:ApoE*$^{-/-}$ *mice influences the APR and aortic plaque macrophage infiltration, but not the development of atherosclerosis*

In light of the above observations, together with conflicting data regarding the effect of artificially-modulating STAT3 activation levels *in vivo* during atherogenesis,[16-19], we genetically defined whether modulating (i.e. normalising) endogenous STAT3 activity in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice would influence high-fat diet-induced atherosclerosis. High-fat diet-fed *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice had a comparable serum lipid metabolism profile, with serum levels of triglycerides remaining similarly reduced in both genotypes compared to *ApoE*$^{-/-}$ mice (Fig. 4A-D). Moreover, the percentage of the aortic root in *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice occupied by atherosclerotic plaques, as well as the total plaque area itself, were comparable to those in

gp130$^{F/F}$:*ApoE*$^{-/-}$ mice and remained significantly lower than *ApoE*$^{-/-}$ mice (Fig. 4E-G). In contrast to these findings, the staining of CD68^{+} macrophages in aortic plaques from *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice was lower than *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice (Supplemental Fig. 2A), and comparable to that observed in parental *ApoE*$^{-/-}$ mice (Fig. 3A). In addition, SAA plasma levels were significantly reduced in *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice compared to *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice (Supplemental Fig. 2B), and were similar to those in *ApoE*$^{-/-}$ mice (Fig. 3C). These data therefore indicate that endogenous gp130/STAT3 signalling modulates the APR and macrophage recruitment processes during high-fat diet-induced atherosclerosis, however these gp130/STAT3-driven processes are insufficient to influence disease pathogenesis in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice.

3.4 Altered gp130 signalling in bone marrow-derived immune cells does not influence atherosclerosis in ApoE-deficient mice

The above data suggest that gp130 signalling in macrophages does not play a critical role in influencing atherosclerotic outcomes. We therefore investigated whether aberrant haemopoietic-specific gp130 signalling in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice influenced their atherogenic hyposensitivity to a high-fat diet. We generated radiation chimeras, whereby *ApoE*$^{-/-}$ mice were lethally irradiated and reconstituted with either heterologous *gp130*$^{F/F}$:*ApoE*$^{-/-}$ (ApoE$^{F/F:ApoE}$) or as a control autologous *ApoE*$^{-/-}$ (ApoE^{ApoE}) bone-marrow cells. Since *gp130*$^{F/F}$ mice spontaneously develop bone marrow-driven splenomegaly and thrombocytosis,[28] spleen weights and circulating platelet counts for both naïve and reconstituted mice were used as patho-physiological indicators to confirm the successful reconstitution of donor bone marrow cells into recipient mice (Supplemental Fig. 3).

Atherosclerotic plaque formation was comparable between both ApoE$^{F/F:ApoE}$ and ApoE^{ApoE} groups of chimeric mice fed a high-fat diet (Fig. 5A-C). Importantly, the percentage of the aortic root occupied by plaques, as well as the size (area) of the plaques, was similar between ApoE^{ApoE} (Fig. 5B,C) and their naïve *ApoE*$^{-/-}$ counterparts (Fig. 1B-D), indicating that the irradiation associated with bone marrow transplantation protocol does not affect atherosclerosis. Serum levels

of triglycerides in high-fat diet-fed ApoE$^{F/F:ApoE}$ mice also remained significantly higher than those in naïve *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice, and were unchanged compared to naïve *ApoE*$^{-/-}$ or ApoE^{ApoE} mice (Fig. 5D). Taken together, these data indicate that alterations to gp130 signals in haemopoietic-derived immune cells from *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice do not influence atherosclerosis.

4. Discussion

Over the last two decades experimental and clinical data have collectively implicated either IL-6, its signalling receptor gp130, or its primary downstream signal-transducing transcription factor STAT3 in influencing the pathogenesis of atherosclerosis. Despite these observations, we are not aware of any study which has previously addressed the role of specific gp130-dependent signalling pathways in disease pathogenesis. Thus, our understanding of the role of the endogenous gp130/STAT3 signalling cascade in modulating the development of atherosclerosis *in vivo* remains unclear. Here, by genetically altering the level of endogenous STAT3 activity in the *gp130*$^{F/F}$ mouse model (on the *ApoE*$^{-/-}$ background) displaying gp130/STAT3 hyper-active signalling, we demonstrate that while gp130/STAT3 hyper-activation augmented both the accumulation of macrophages in aortic lesions and the systemic APR (as measured by increased plasma SAA production) during high-fat diet-induced atherosclerosis, this alone was not sufficient to promote the development of aortic plaques. Indeed, this latter observation is supported by the recent finding that SAA deficiency in *ApoE*$^{-/-}$ mice has no effect on the development of aortic lesions[29]. Rather, we reveal here that *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice were protected against the development of atherosclerotic lesions in a gp130/STAT3-independent manner, which was associated with a specific decrease in plasma triglyceride levels, the latter observation consistent with clinical data suggesting that lower plasma triglyceride levels correlate with reduced risk of cardiovascular disease.[5]

The role of STAT3 in experimentally-induced atherosclerosis is controversial, and has largely relied on methods to either directly modulate the expression levels of total STAT3 or indirectly alter the activation status of STAT3, the latter by modulating the expression levels of SOCS3 which upon association with specific receptors (such as gp130) suppresses receptor-mediated STAT3 activation. For instance, an anti-atherogenic role for STAT3 has been suggested from the demonstration that adenoviral-mediated gene delivery of wild-type STAT3 to *Ldlr*$^{-/-}$ mice fed a high-fat diet suppressed atherosclerosis with a concomitant reduction in lesion-associated macrophage infiltrates.[16] In addition, the conditional ablation of *Socs3* in T cells augmented STAT3

activation which correlated with a reduction in the development of atherosclerotic lesions, albeit without any changes in the numbers of infiltrating macrophages.[18] Conversely, STAT3 has been proposed to promote atherogenesis, as evidenced by the administration of *ApoE*$^{-/-}$ mice with anti-sense oligonucleotides against SOCS3 which augmented STAT3 activity and led to increased aortic plaque development and macrophage infiltration.[17] Furthermore, genetic ablation of *Stat3* in the endothelial and, to a lesser extent, the hematopoietic compartments ameliorated both the formation of atherosclerotic lesions and the associated macrophage content in response to a high-fat diet, suggesting that STAT3 activation in multiple cell types can contribute to disease pathogenesis.[19] Indeed, this notion is supported by clinical studies investigating the expression of activated STAT3 in human atherosclerotic lesions collected by carotid endarterectomy, which indicate that STAT3 is activated in a wide variety of cell types within human lesions, including endothelial, inflammatory (macrophages, T cells) and vascular smooth muscle cells.[18,19] Notably, such clinical data have also further highlighted the contrasting atherogenic roles assigned to STAT3, with one study revealing that high STAT3 activity (anti-atherogenic) is associated with lesions containing low macrophage (i.e. inflammatory cell) numbers,[18] while conversely another study indicated a positive correlation between high STAT3 activation levels (pro-atherogenic) and lesions with high grade inflammation.[19]

While the opposing atherogenic roles assigned to STAT3 during the above-mentioned experimentally-induced models of atherosclerosis could be explained, at least in part, by experimental differences including the mouse strains (*ApoE*$^{-/-}$, *Ldlr*$^{-/-}$) and diet used, it is important to note that the differential production of STAT3-activating cytokines during these experimentally-induced atherosclerosis models, and for that matter human disease, is likely to influence whether STAT3 suppresses or promotes disease pathogenesis. In this regard, the anti-atherogenic role proposed for STAT3 has been in the context of facilitating the anti-inflammatory actions of IL-10 and/or IL-17 which were up-regulated during disease pathogenesis, although a potential direct link between IL-10/STAT3 and/or IL-17/STAT3 in these atherosclerosis models was not

investigated.[16,18] The anti-atherogenic actions of IL-17 were assigned to the dampening of T cell infiltration into forming lesions[18], which contrasts its reported pro-atherogenic role via its ability to drive the infiltration of pro-atherogenic myeloid cells to atherosclerotic aortas.[30] While other STAT3-activating cytokines such as leptin have also been implicated in atherosclerosis, STAT3 activation via the leptin receptor (LEPR) is unlikely to contribute to atherosclerosis since plaque formation (including the presence of aortic macrophages) was unaltered in *ApoE*$^{-/-}$ mice expressing a LEPR mutant unable to elicit STAT3 signalling.[31] With respect to the role of gp130-activating cytokines during atherosclerosis, while IL-6 is the most widely investigated, its genetic ablation, transgenic over-expression or exogenous administration in atherosclerosis-prone mouse backgrounds have led to conflicting findings regarding its pro- or anti-atherogenic properties[9,12,13,32]. While the mechanistic basis for these diverse findings remain ill-defined, they reflect the complex spectrum of atherogenic-related cellular processes affected by IL-6 signalling, including lipid homeostasis, metabolism, vascular remodelling, and the recruitment and activation of inflammatory cells, the latter of which can manifest as either anti- or pro-inflammatory.

Our data presented here raises the question as to which gp130 signalling pathway(s) independent of STAT3 influence atherosclerotic plaque formation. In light of the observed protection against atherosclerosis in *gp130*$^{F/F}$ mice, one would suggest the involvement of either suppressed pro-atherogenic or enhanced anti-atherogenic signalling from the mutant gp130 receptor. Such a pathway is unlikely to involve PI3K/Akt whose activation from gp130 is suppressed in *gp130*$^{F/F}$ mice, since the modulation of PI3K signalling in *ApoE*$^{-/-}$ mice on a PTEN-deficient background had no effect on high-fat diet-induced atherosclerosis[33]. In addition, genetic ablation of *Akt1* or *Akt3* isoforms in *ApoE*$^{-/-}$ mice has indicated an athero-protective role for Akt signalling in either the bone marrow or vascular compartments[34,35]. The notion that the absence of specific pro-atherogenic gp130 signalling cascades in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice may be responsible for the reduced plaque formation is supported by the observation that *ApoE*$^{-/-}$ mice harbouring hepatic-specific conditional deletion of gp130 are also protected against atherosclerosis[10]. In this regard, while

studies with inhibitors against mammalian target of rapamycin (mTOR) in atherosclerotic *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mouse models have implicated a pro-atherogenic role for this pathway[36, 37], we note that mTOR is over-activated in *gp130*$^{F/F}$ mice[38].

In summary, our current study provides further evidence *in vivo* for the importance of the gp130 cytokine receptor in modulating the development of atherosclerotic lesions. Furthermore, we identify that the gp130/STAT3 signalling axis plays a key role in directing the macrophage infiltration into aortic lesions and the acute phase response during atherosclerosis; however, these processes alone are not sufficient to influence disease pathogenesis. These data therefore implicate gp130/STAT3-independent signalling pathways in promoting the molecular pathogenesis of atherosclerotic disease, the future identification of which will further broaden our understanding of the complexities by which gp130-activating cytokines contribute to cardiovascular disease.

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Conflicts of interest

The authors have no conflicts to declare.

Figure Legends

Figure 1

Suppressed atherosclerosis in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice mice fed a high-fat diet. (A) Weight of *ApoE*$^{-/-}$ (n=9) and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ (n=9) mice at the beginning (week 0) and end (week 10) of being fed a high-fat diet. (**B**) Representative photomicrographs showing aortic root cross-sections stained with oil red O in *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice after 10 weeks of being fed a high-fat diet. Arrows point to representative red-stained areas containing atherosclerotic plaques. Scale bars=200μm. (**C, D**) Quantitative analysis of oil red O-stained plaques as (C) a percentage within the whole aortic root and (D) total surface area in the aortic root. Data are from n=6 *ApoE*$^{-/-}$ and n=5 *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice, and are expressed as mean±SEM. ***P<0.001.

Figure 2

Reduced systemic levels of triclycerides in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice fed a high-fat diet. *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice were fed a high-fat diet for 10 weeks, following which serum concentrations were measured for (**A**) triglycerides, (**B**) total cholesterol, (**C**) HDL cholesterol, and (**D**) LDL cholesterol. n=12 mice per genotype. Data are presented as the mean±SEM. **P<0.01.

Figure 3

Augmented aortic plaque macrophage infiltration and production of SAA in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice fed a high-fat diet. (**A**) Left panels; representative photomicrographs showing aortic root cross-sections stained with CD68 antibody in *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice after 10 weeks of being fed a high-fat diet. Scale bars=100μm. Right panels; magnified area of the aortic root with CD68 staining. Scale bars=50μm. (**B**) Quantitative analysis of CD68^{+} cells within plaques expressed as a percentage of the total plaque surface area. Data are from n=4 *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice, and are expressed as mean±SEM. **P<0.01. (**C**) ELISA of SAA protein

levels in the plasma of high fat diet-fed *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice. Data are from n=4 *ApoE*$^{-/-}$ and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice, and are expressed as mean±SEM. **P*<0.05.

Figure 4

Activation of the gp130/STAT3 signalling axis does not influence atherosclerosis in *gp130*$^{F/F}$ mice. (A-D) Mice of the indicated genotypes were fed a high-fat diet for 10 weeks, following which serum concentrations were measured for (**A**) triglycerides, (**B**) total cholesterol, (**C**) HDL cholesterol, and (**D**) LDL cholesterol. n=5-6 mice per genotype. Data are presented as the mean±SEM. **P*<0.05. **(E)** Representative photomicrographs showing aortic root cross-sections stained with oil red O in mice of the indicated genotypes after 10 weeks of being fed a high-fat diet. Arrows point to representative red-stained areas containing atherosclerotic plaques. Scale bars=200μm. (**F, G**) Quantitative analysis of oil red O-stained plaques as (F) a percentage within the whole aortic root and (G) total surface area in the aortic root. Data are from n=4 mice per genotype, and are expressed as mean±SEM. **P*<0.05.

Figure 5

Altered gp130 signalling in *gp130*$^{F/F}$ bone marrow-derived immune cells does not influence atherosclerosis in ApoE-deficient mice. (**A**) Representative photomicrographs showing aortic root cross-sections stained with oil red O in recipient *ApoE*$^{-/-}$ mice reconstituted with bone marrow from *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice (ApoE$^{F/F:ApoE}$) or *ApoE*$^{-/-}$ mice (ApoE^{ApoE}) after 10 weeks of being fed a high-fat diet. Arrows point to representative red-stained areas containing atherosclerotic plaques. Scale bars=200 μm. (**B, C**) Quantitative analysis of oil red O-stained plaques as (B) a percentage within the whole aortic root and (C) total surface area in the aortic root. Data are from n=4 ApoE^{ApoE} and n=5 ApoE$^{F/F:ApoE}$ mice. (**D**) Serum concentrations of triglycerides in naïve *ApoE*$^{-/-}$ (n=6) and *gp130*$^{F/F}$:*ApoE*$^{-/-}$ (n=6) mice, as well as ApoE$^{F/F:ApoE}$ (n=5) and ApoE^{ApoE} (n=4) mice, after 10 weeks fed on a high-fat diet. Data are presented as the mean±SEM. **P*<0.05, ***P*<0.01.

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***Statement of Originality**

July 3, 2014

Dear Editor,

On behalf of all authors of our manuscript entitled "Modulation of atherosclerosis in ApoE-deficient mice by gp130 signalling independent of STAT3" by Gareth Jones and colleagues, I hereby confirm that we have met all of the necessary criteria regarding originality of this work.

Kind regards,

Professor Brendan J. Jenkins, PhD (Med)

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***Submission Letter Suggested Reviewers**

July 3, 2014

Dear Editor,

Please find enclosed our manuscript entitled "Modulation of atherosclerosis in ApoE-deficient mice by gp130 signalling independent of STAT3" by Gareth Jones and colleagues for your consideration for publication in *Atherosclerosis*.

The gp130 cytokine receptor transduces signals from a large number of cytokines, known as the interleukin (IL)-6 cytokine family. Among these cytokines IL-6 is a potent modulator of many atherogenic-related processes including lipid homeostasis, metabolism, vascular remodelling, the acute phase response, and the recruitment and activation of inflammatory cells, the latter of which can manifest as either anti- or pro-inflammatory. However, the pathological role of specific IL-6/gp130 signalling cascades during atherosclerosis is ill-defined.

IL-6 primarily activates the latent transcription factor STAT3 via gp130, and STAT3 itself has been implicated in atherosclerosis, albeit with controversial findings. This is largely due to different *in vivo* approaches to artificially modulate STAT3 activity, either indirectly by targeting the negative cytokine signalling regulator SOCS3 (which can also interfere with other pathways) or directly by modulating STAT3 expression levels. In addition, such approaches do not address which upstream cytokine receptor system is responsible for activation of STAT3 during disease.

To address these issues and advance our fundamental understanding of the complex molecular and cellular basis by which the gp130 receptor influences atherosclerosis, we report here the use

of a genetic approach with the *gp130*$^{F/F}$ knock-in mutant mouse characterized by hyper-activation of endogenous STAT3 to define whether endogenous gp130-dependent STAT3 activation modulates atherosclerosis.

Specific novel findings of our current manuscript are as follows:

1) aortic plaque development and plasma triglyceride levels were significantly reduced in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice fed a high-fat diet.

2) the reduced atherosclerosis in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice surprisingly correlated with augmented CD68^{+} macrophage infiltrates in the smaller atherosclerotic plaques, and plasma serum amyloid A (as a read-out for the acute phase response) levels were elevated.

3) genetic reduction of endogenous STAT3 activity in *gp130*$^{F/F}$:*Stat3*$^{-/+}$:*ApoE*$^{-/-}$ mice had no effect on atherosclerotic lesion development and plasma triglyceride levels, despite macrophage numbers in lesions and plasma SAA levels being significantly reduced back to wild-type levels.

4) bone marrow chimeras revealed that the suppressed aortic plaque development and plasma triglyceride levels in *gp130*$^{F/F}$:*ApoE*$^{-/-}$ mice were independent of gp130 signals in bone marrow (immune) cells.

Thus, our current study provides new evidence *in vivo* for the importance of the gp130 cytokine receptor in modulating the development of atherosclerotic lesions. Furthermore, we also identify that while the endogenous gp130/STAT3 signalling axis plays a key role in directing the macrophage infiltration into aortic lesions and the acute phase response during atherosclerosis, these processes alone are not sufficient to influence disease pathogenesis. We believe these novel data therefore implicate gp130/STAT3-independent signalling pathways in promoting the molecular pathogenesis of atherosclerotic disease, the future identification of which will further

broaden our understanding of the complexities by which gp130-activating cytokines contribute to atherosclerosis. In the burgeoning international climate of intense research into cardiovascular disease, as well as the role of cytokine signalling in general disease pathogenesis, we believe our novel mechanistic insights presented here are most timely and appropriate for dissemination among the broad readership of *Atherosclerosis* including molecular and cell biologists, as well as clinical researchers.

Potential reviewers with particular expertise in the areas of atherosclerosis, the immune system and gp130 signalling include the following:

Eicke Latz, expert in atherosclerosis and the immune system; Institute of Innate Immunity, University of Bonn, Germany; eicke.latz@uni-bonn.de

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The authors declare that they have no conflict of interest.

We also note that all authors are in agreement with submission of the manuscript to *Atherosclerosis*.

We look forward to hearing from you at your earliest convenience.

Kind regards,

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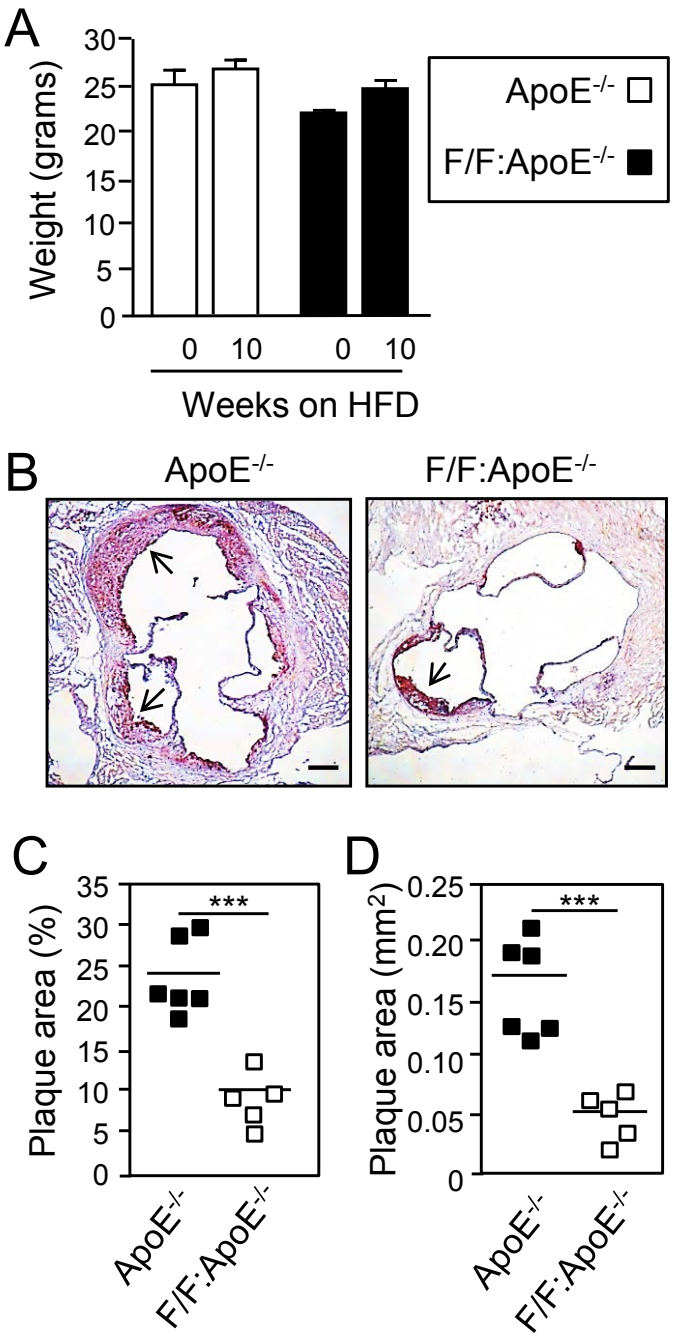
Figure 1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20
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10
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0
0
10
0
10
Weeks on HFD
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F/F:ApoE-/-
B
ApoE-/-
F/F:ApoE-/-
C
Plaque area (%)
35
30
25
20
15
10
5
0
***
ApoE-/-
F/F:ApoE-/-
D
Plaque area (mm2)
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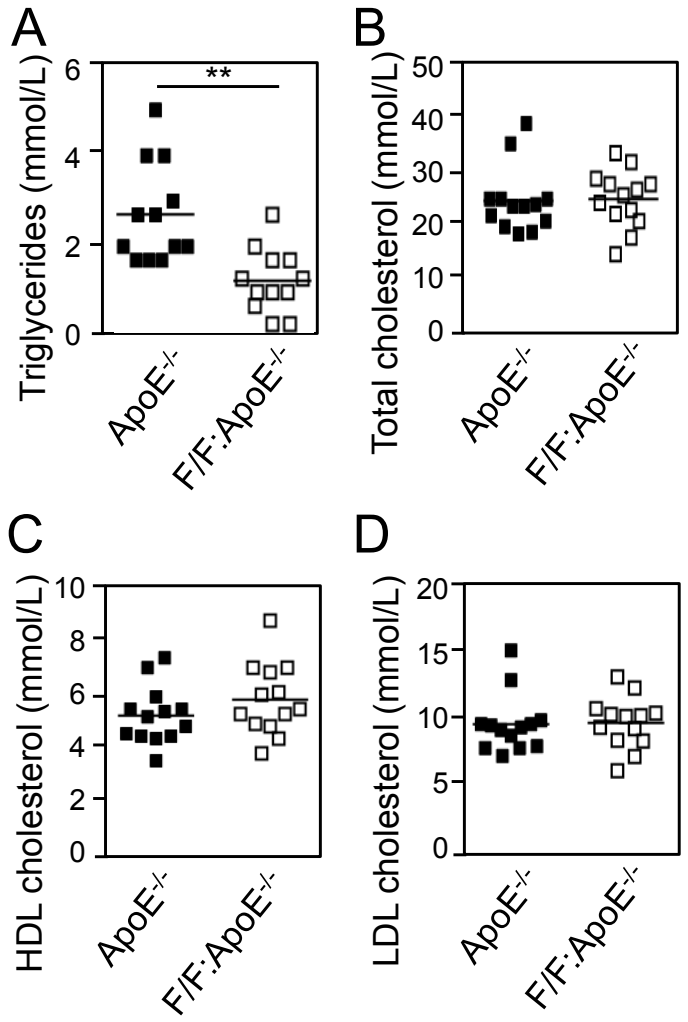
0.15
0.10
0.05
0
***
ApoE-/-
F/F:ApoE-/-

Figure 2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4
2
0
**
ApoE-/-
F/F:ApoE-/-
B
Total cholesterol (mmol/L)
50
40
30
20
10
0
ApoE-/-
F/F:ApoE-/-
C
HDL cholesterol (mmol/L)
10
8
6
4
2
0
ApoE-/-
F/F:ApoE-/-
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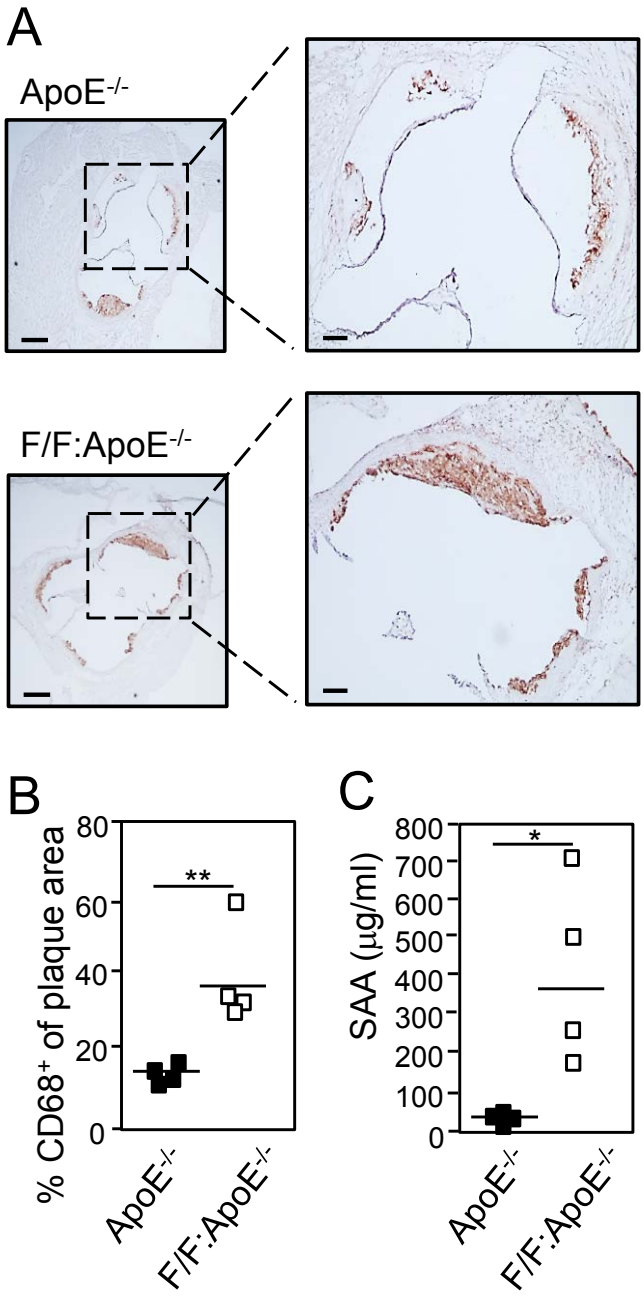
10
5
0
ApoE-/-
F/F:ApoE-/-

Figure 3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ApoE-/-
F/F:ApoE-/-
B
% CD68+ of plaque area
80
60
40
20
0
**
ApoE-/-
F/F:ApoE-/-
C
SAA (μg/ml)
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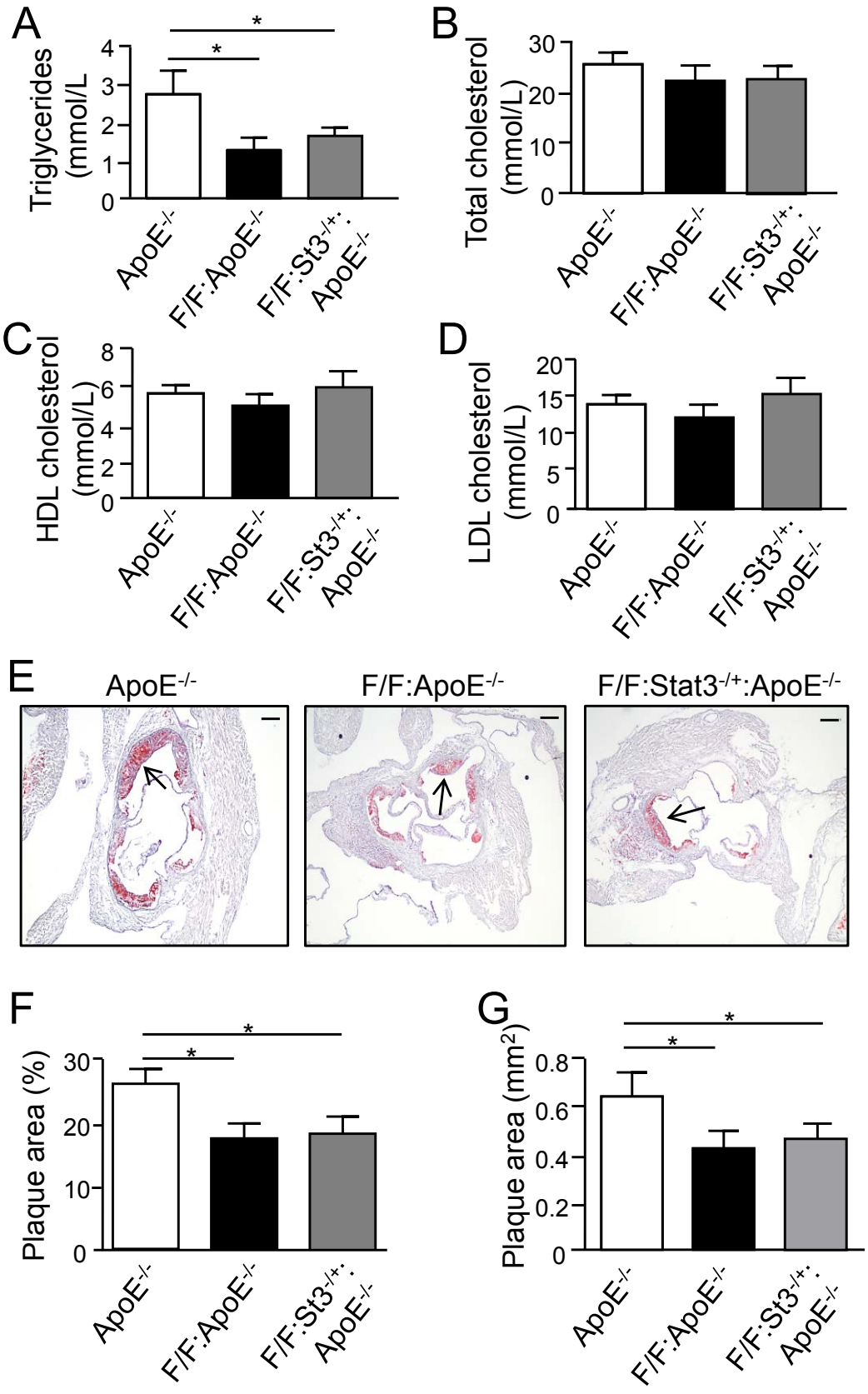
700
600
500
400
300
200
100
0
*
ApoE-/-
F/F:ApoE-/-

Figure 4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Triglycerides (mmol/L
4
3
2
1
0
*
*
ApoE-/-
F/F:ApoE-/-
F/F:St3-/+:ApoE-/-
B
Total cholesterol (mmol/L)
30
20
10
0
C
HDL cholesterol (mmol/L)
8
6
4
2
0
D
LDL cholesterol (mmol/L)
20
15
10
5
0
E
ApoE-/-
F/F:ApoE-/-
F/F:Stat3-/+:ApoE-/-
F
Plaque area (%)
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20
10
0
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Plaque area (mm2)
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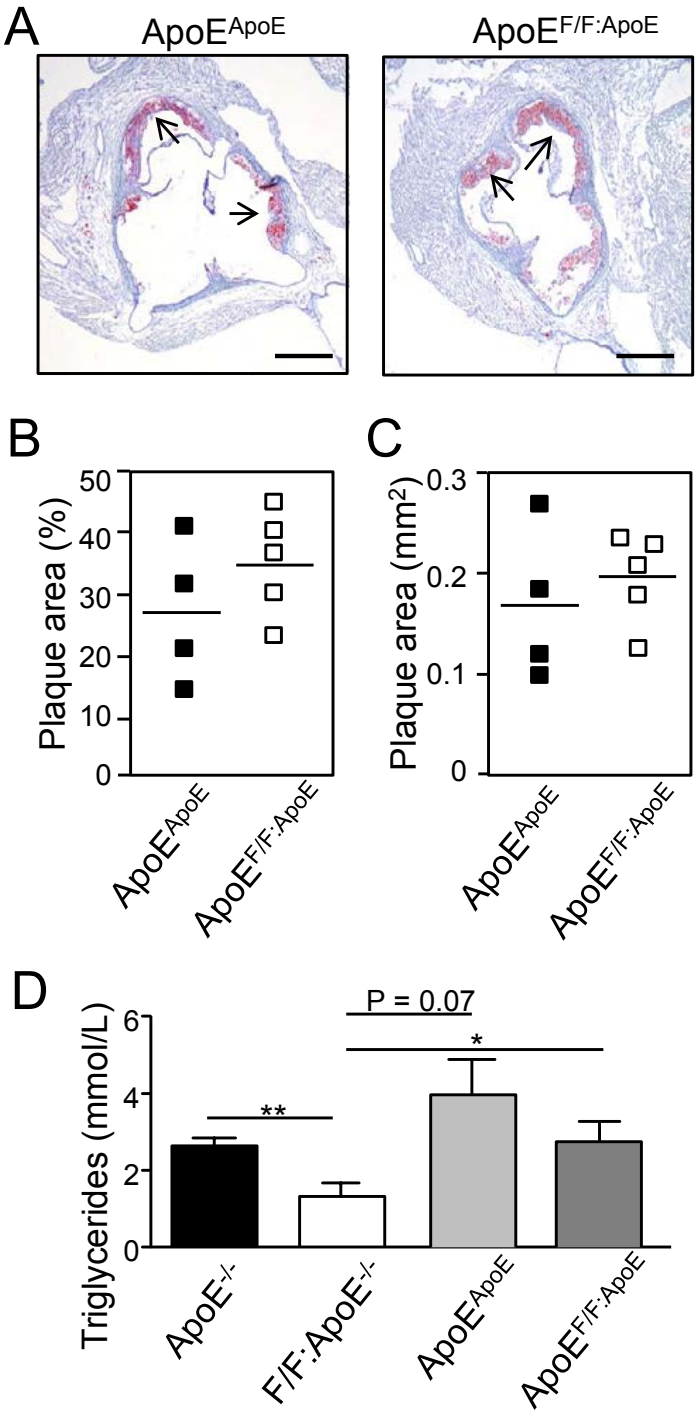
0.4
0.2
0

Figure 5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ApoE^ApoE
ApoE^F/F:ApoE
B
Plaque area (%)
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40
30
20
10
0
ApoE^ApoE
ApoE^F/F:ApoE
C
Plaque area (mm²)
0.3
0.2
0.1
0
ApoE^ApoE
ApoE^F/F:ApoE
D
Triglycerides (mmol/L)
6
4
2
0
P = 0.07
*
**
ApoE^-/-
F/F:ApoE^-/-
ApoE^ApoE
ApoE^F/F:ApoE